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MOLLUSCUM CONTAGIOSUM: NORMAL AND VIRUS INFECTED EPIDERMAL CELL KINETICS*

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Molluscum contagiosum offers an unparalleled opportunity to study directly the effects of tumor virus infection on cell division and differentiation in man. In this paper we examine the effect of virus infection on human epidermis using thymidine-H^3 and cytidine-H^3 to label viral and cellular DNA and RNA. The results allow consideration of two noteworthy findings: 1) viral infection and replication in molluscum contagiosum *in vivo*, and 2) how epidermal cells relate to each other during differentiation.

MATERIALS AND METHODS

Cell renewal and function were assessed by labeling DNA with thymidine-H^3, RNA with cytidine-H^3 and arresting mitoses in metaphase with Colcemid. Eight persons with multiple lesions of molluscum contagiosum served as subjects. Ten microcuries (μc) of thymidine-H^3 were injected intradermally into lesions and biopsy specimens secured 40 minutes to one month later (1). Cytidine-H^3 (10 μc) was also injected intradermally and biopsies obtained in 30 minutes. All specimens were fixed in buffered neutral formalin and processed for film dipping radioautography (2, 3). As cytidine is incorporated into RNA and DNA (4), the radioautographs were controlled with DNase[1] and by hot acid hydrolysis to remove RNA (5). In addition, newly formed RNA is recognized by the characteristic pattern of cytidine-H^3 labeling of nucleoli (5, 6). Colcemid, 300 μg, was injected intradermally (7). Biopsy specimens were taken in 2–3 hours. Sections were stained with hematoxylin and eosin, Giemsa, and by the Feulgen method.

Cell renewal in the germinative layer was estimated employing the appropriate formula[2] after counting the number of thymidine-H^3 labeled or metaphase figures in 3,000 to 5,000 basal cells (1, 8, 9, 10). Tritium-injected specimens were examined by light and phase microscopy for relative number and distribution of silver grains.

RESULTS AND COMMENT

In molluscum contagiosum, virus-laden cells lay embedded in a central horny mass formed by uninfected cells intimately related to infected cells in the granular and Malpighian layers (11–14). The different cells are readily distinguished in Feulgen-treated tissue by the compressed, dark-staining, moth-eaten nucleus of infected cells and the larger, more delicate staining, relatively intact nucleus of keratinforming cells (Fig. 1). We counted 1,000 such cells at random and considered 309 (31%) uninfected and 691 (69%) infected. Infected cells became greatly distended with virus, compressing more normal cells. They occupied 90 per cent of space in the Malpighian layer. Observation of the Feulgen stained basal cells and those just above gave no clue to which cell would become infected.

Early thymidine-H^3 and cytidine-H^3 labeling

In thymidine-H^3 injected lesions at 40 minutes nuclear labels were distributed solely in

[2] *Thymidine-H^3 method:*

$$ {}_BT = \frac{t_S}{n_S/{}_BN} $$

(${}_BT$ = turnover time of the germinative cell layer; t_S = DNA synthesizing time. This is taken at 7 hours (1); $n_S/{}_BN$ = fraction of labeled cells to total cells in the germinative layer).

Colcemid method:

$$ {}_BT = \frac{100\%}{Rm/24 \text{ hr.}} $$

(${}_BT$ = turnover time of the germinative cell layer; $Rm/24$ hr. = mitotic rate per 24 hours).

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[1] Purchased from Worthington Chemical Company and used fresh.

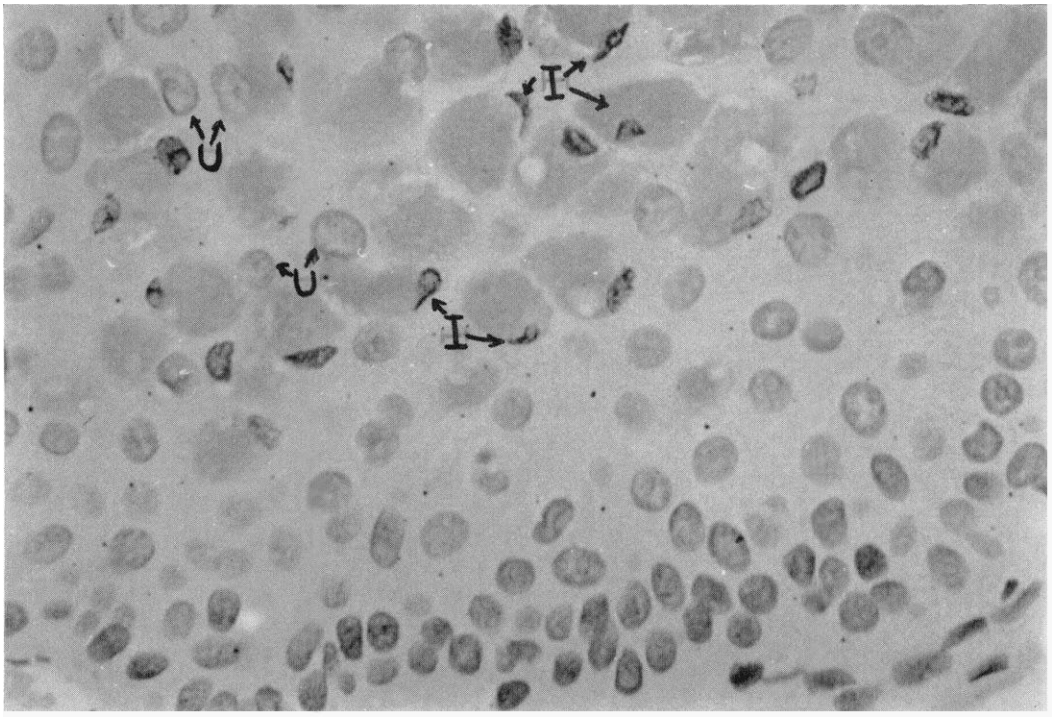


FIG. 1. A lobule of a molluscum contagiosum lesion stained by the Feulgen method. Nuclei in the basal layer and one or two layers above seem normal. Then large light-staining cytoplasmic inclusions appear and the nuclei of these cells (I) become compressed, dark-staining, and "moth-eaten." Contrast these with nuclei of uninfected cells (U).

basal cells (Fig. 2a). The pattern was random and mimicked normal human epidermis (1), except more cells contained labels. Cytoplasmic labeling occurred over all viral inclusion bodies in the Malpighian layer. Labeling appeared most intense in the more basal cells and followed a gradient of diminished labeling toward the granular layer (Fig. 2b). In some lesions labeled inclusions occurred in the lower granulay layer. DNase treatment removed tritium labeling from both inclusion bodies and basal nuclei (Fig. 7a and b). Labeled inclusions were not detected in the upper granular or horny layers in 40 minute specimens, indicating that the virus stops replicating in or below the granular layer. More important, we observed cytoplasmic labeling only in cells with inclusion bodies. No nuclear or cytopasmic labeling preceded the appearance of inclusions visible by light microscopy. In other words, the virus makes its presence known suddenly and without warning.

In cytidine-H^3 injected lesions at 40 minutes nearly 90 per cent of basal nuclei contained labels. These were concentrated in and about nucleoli (Fig. 3a), following the pattern of early labeling RNA seen in other epidermal tissues (15, 16). Nearly all the labeling was insensitive to DNase and was removed by hot acid hydrolysis (5), indicating little or no cytidine-H^3 had been incorporated into DNA in the 40 minute specimens. Cells in the Malpighian and granular layers showed a very different pattern. Uninfected cells took up cytidine-H^3 in the usual way, but almost none of the nuclei of infected cells contained any label; a few with early inclusions showed nuclear labeling. On the other hand, many cytoplasmic inclusions up to the granular layer possessed some labeling that was sensitive to hot acid hydrolysis and presumably was viral directed RNA (Fig. 3b). This never was great and disappeared below the granular layer. We could not, however, accurately assess the amount of viral RNA synthesis per cell. In essence, virus infection of the cell shuts off

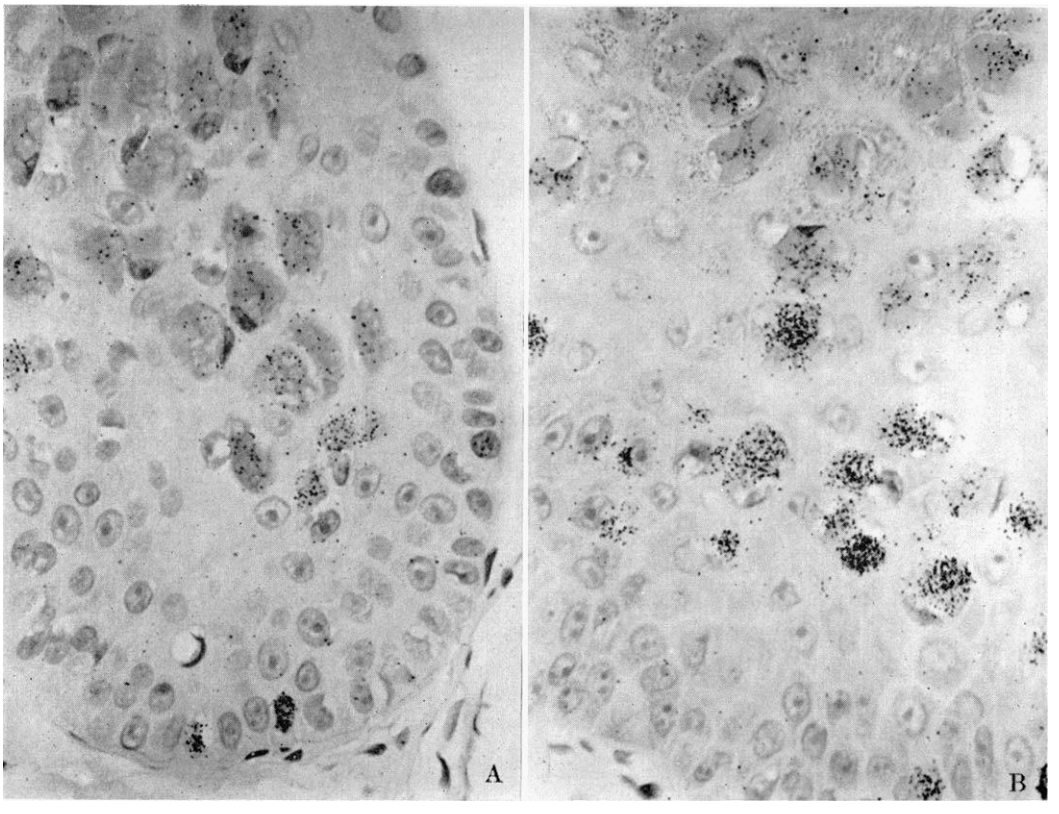


FIG. 2a. Radioautograph of Feulgen treated tissue 40 minutes after intradermal injection of 10 uc. thymidine-H^3. Note random nuclear labeling restricted to the basal layer and cytoplasmic labeling over inclusions.

FIG. 2b. Radioautograph 40 minutes after thymidine-H^3 injection. Note gradient of density of labeling over inclusion extending from basal regions to the granular layer. No cytoplasmic labeling is seen in outer granular and horny layers.

nuclear direction of RNA synthesis and assumes this function with perhaps less intensity in the cytoplasmic inclusion. Again, the important link to "uninfected" basal cells was not discovered. We observed few basal nuclei devoid of labeling and no cytoplasmic labeling occurred in the absence of a visible inclusion.

Cell renewal

Cell renewal in the germinative layer was estimated by the method of Leblond *et al* (8) in 6 specimens secured 40 minutes after thymidine-H^3 injection (Table I). The average renewal time of 3.4 days is almost half that for normal human epidermis (6.1 days) reported previously (1). In 5 other specimens germinative cell renewal estimated by the Colcemid method (9, 10) gave an average renewal time of 2.8 days (Table II). Despite a minor discrepancy the results by both methods clearly indicate that virus infection causes a more rapid appearance of new germinative cells. What happens to these cells?

Fate of thymidine-H^3 labeled nuclei and virus

The fate of thymidine-H^3 labeled cells was followed in timed biopsy specimens. At 24 hours after thymidine-H^3 injection, labeled nuclei had almost doubled (1). A few had moved out of the basal layer (Fig. 4a). By 48 hours more labeled nuclei had moved out of the basal layer, but none had reached the level of infected cells. On the whole, movement appeared sluggish. In the meantime, cells with labeled inclusions had moved swiftly. In 24 hour specimens labeled inclusions appeared in the lowermost keratin layer (Fig. 4b). At the same time all viral inclusions in the base of the lesion contained

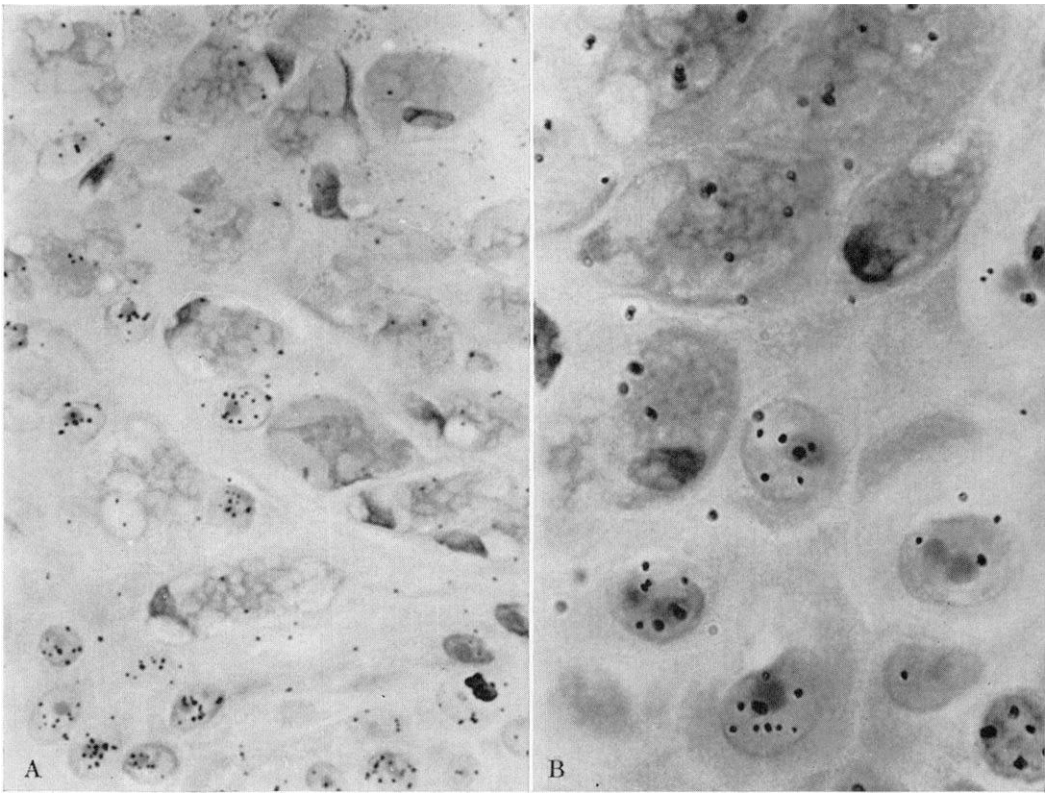


FIG. 3a. Radioautgoraph 40 minutes after cytidine-H^3 injection. Most nuclei in basal area contain labels and they are concentrated about the nucleolus. This is the typical pattern of early labeled RNA in epidermal tissues. Note also lack of labeling over nuclei of infected cells.

FIG. 3b. High power similar to 3a and showing nucleolar labeling of uninfected cells and essentially no labeling of nuclei in infected cells. Some labeling is seen over inclusions. It was not great in most specimens (see 3a).

TABLE I

Germinative cell renewal—thymidine-H^3 method (8)

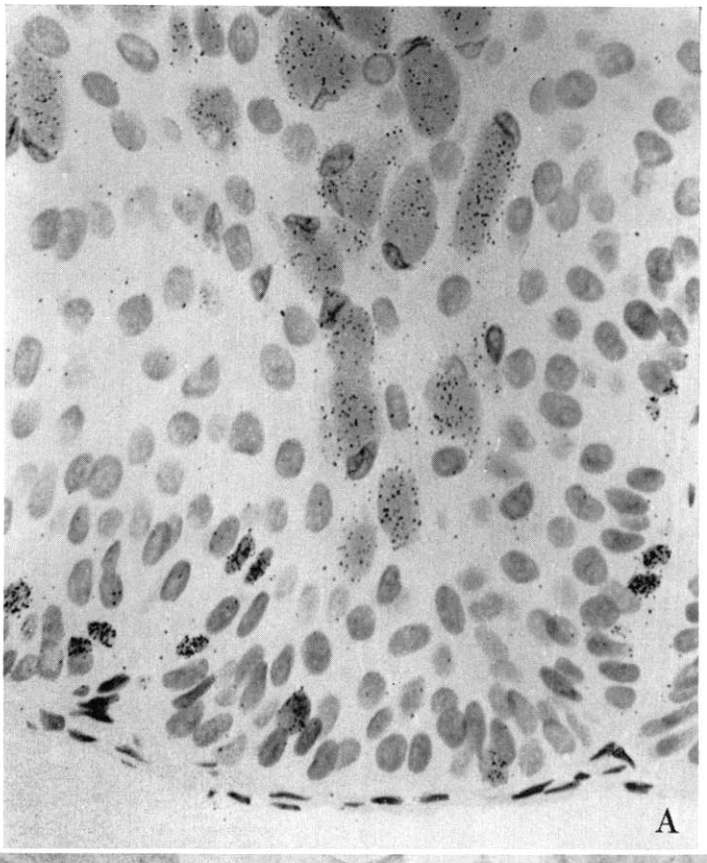
Subject	N_S	$_BN$	$_BT$ (days)
1	231	3,000	3.8
2	207	2,988	4.2
3	250	2,926	3.4
4	400	3,199	2.3
5	234	3,000	3.7
6	316	3,000	2.8
Average			3.4

TABLE II

Germinative cell renewal—colchicine method (9, 10)

Subject	Time Colcemid Acted (hours)	Metaphase Figures/1,000 Cells	Mitotic Rate/Hr (per cent)	$_BT$ (days)
1	3.0	40.8	1.36	2.9
2	2.0	30.0	1.5	3.2
3	2.0	41.2	2.06	2.0
4	2.5	32.5	1.3	2.8
5	2.5	36.1	1.44	3.1
Average				2.8

labels (Fig. 4a). By 48 hours newly formed cytoplasmic inclusions free of label were seen for the first time. At this time the outer band of labeled cells had moved well into the horny layer. The lower border of labeled inclusions had also moved up in a band-like fashion. This was very striking in 5 day old specimens in which a wide band of labeled inclusions was restricted to the mid- and lower keratin layer and sometimes the upper granular layer (Fig.



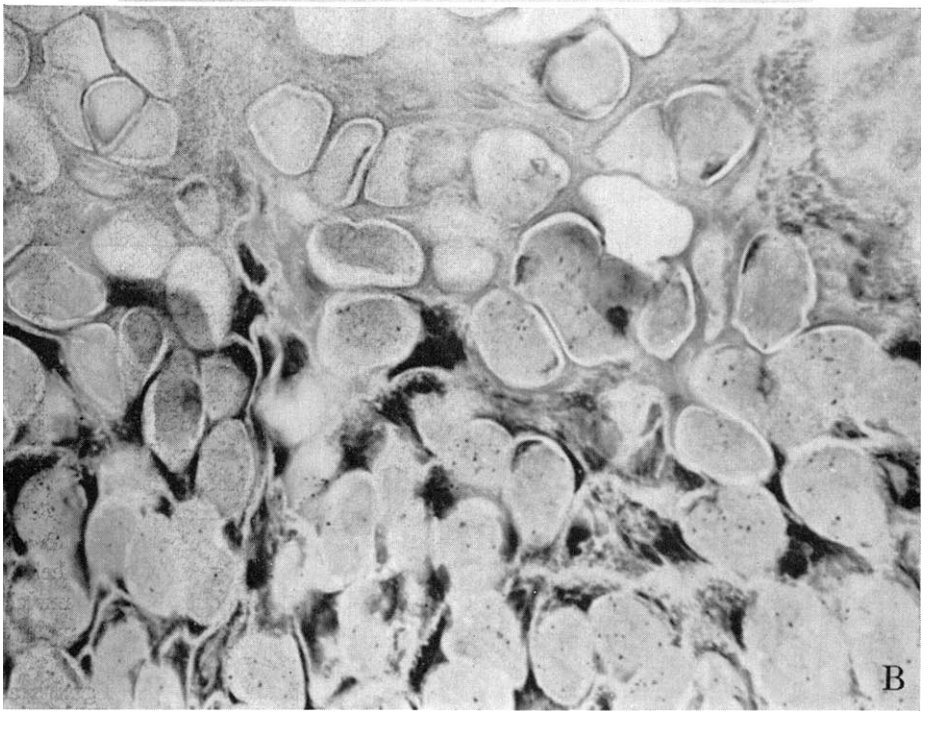


FIG. 4a. Radioautograph 24 hours after thymidine-H^3 injection. Note pairing of labeled nuclei and the fact no visible inclusions are free of labels. Feulgen method.

FIG. 4b. Radioautograph similar to 4a shows labeled inclusions in the lowermost horny layer. H and E.

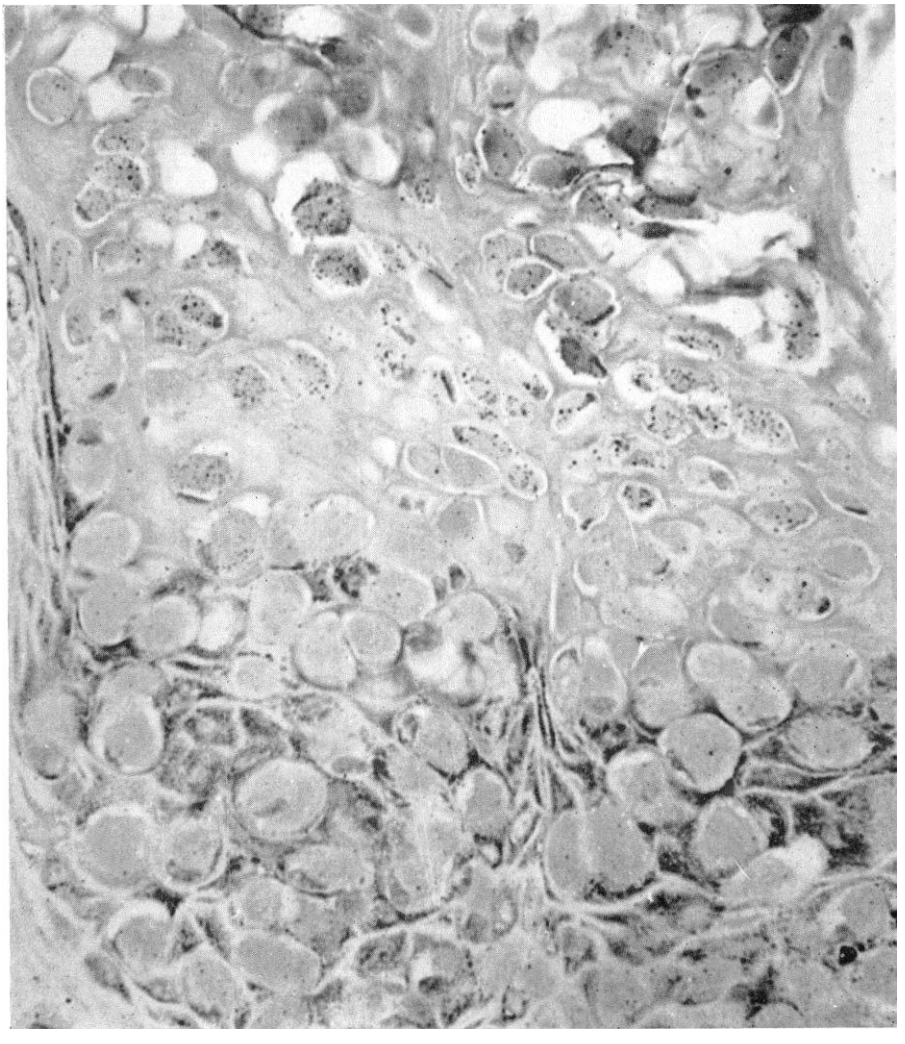
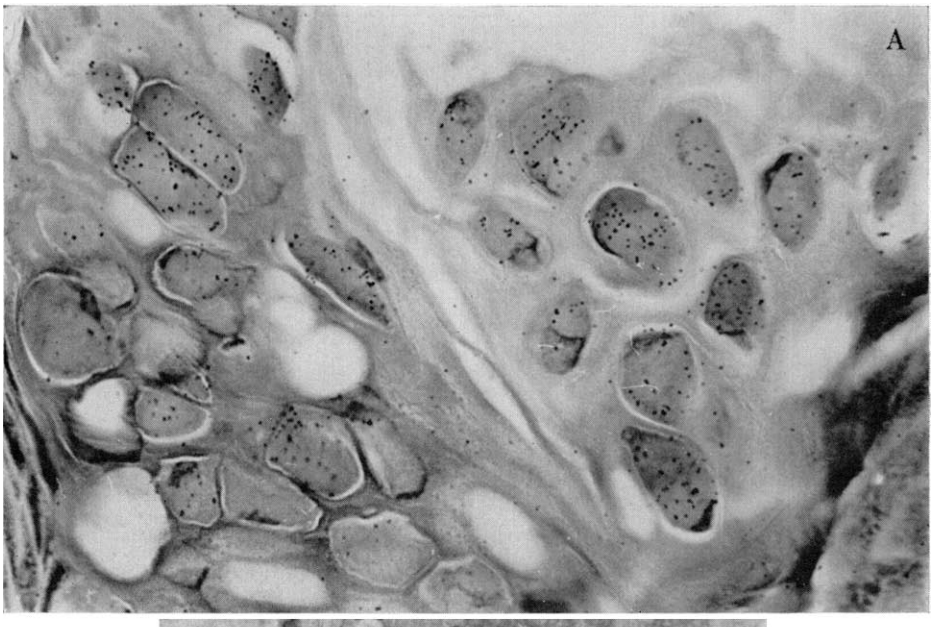


FIG. 5. Radioautograph of H and E stained tissue 5 days after injection of thymidine-H^3. A band of labeled inclusions 12 to 14 cells thick appears in the lower and mid horny layer.

5). The amount of downward diffusion of less intensely labeled inclusions was minimal. If virus is transferred from one cell to the next, it is not apparent. After 7 days the band of labeled inclusions had moved to the mid- and outermost horny layers (Fig. 6a and b). At this time labeled inclusions resisted the action of DNase (Fig. 7c and d). Between 9 and 15 days after injection, the band of labeled virus disappeared at the surface. In one 9 day specimen the pasty material that often extrudes from the surface during biopsy was fixed and prepared for radioautography. It contained disintegrating cells and an amorphous mass labeled with tritium, presumably free virus particles (Fig. 8). At the same time labeled nuclei of both infected and uninfected cells were seen in the Malpighian and lower granular layers. Very few labeled germinative cells were left (Fig. 9). The movement of cells seemed random and haphazard. The pattern of lebeling gave no clue as to which cell would become infected. By 3 weeks labeled nuclei had moved farther out. Labeled nuclei of infected cells appeared in the horny layer. The compressed and moth-eaten nuclei of infected cells contained about as many grains as uninfected cells; there was no evidence of transfer of nuclear label to cytoplasmic inclusions. Ap-



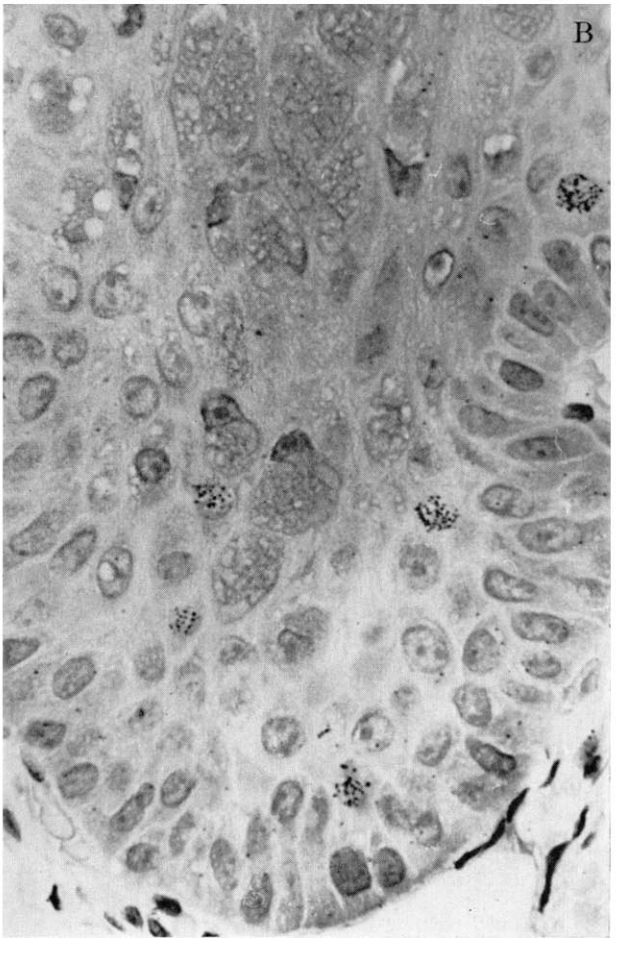


FIG. 6a. Labeled inclusions in the outermost horny layer 8 days after injection of thymidine-H^3. The time for disappearance of these cells ranged from 9 to 15 days.

FIG. 6b. Basal portion of molluscum lesion 8 days after thymidine-H^3 injection shows lack of labeled inclusions and very gradual movement of labeled nuclei.

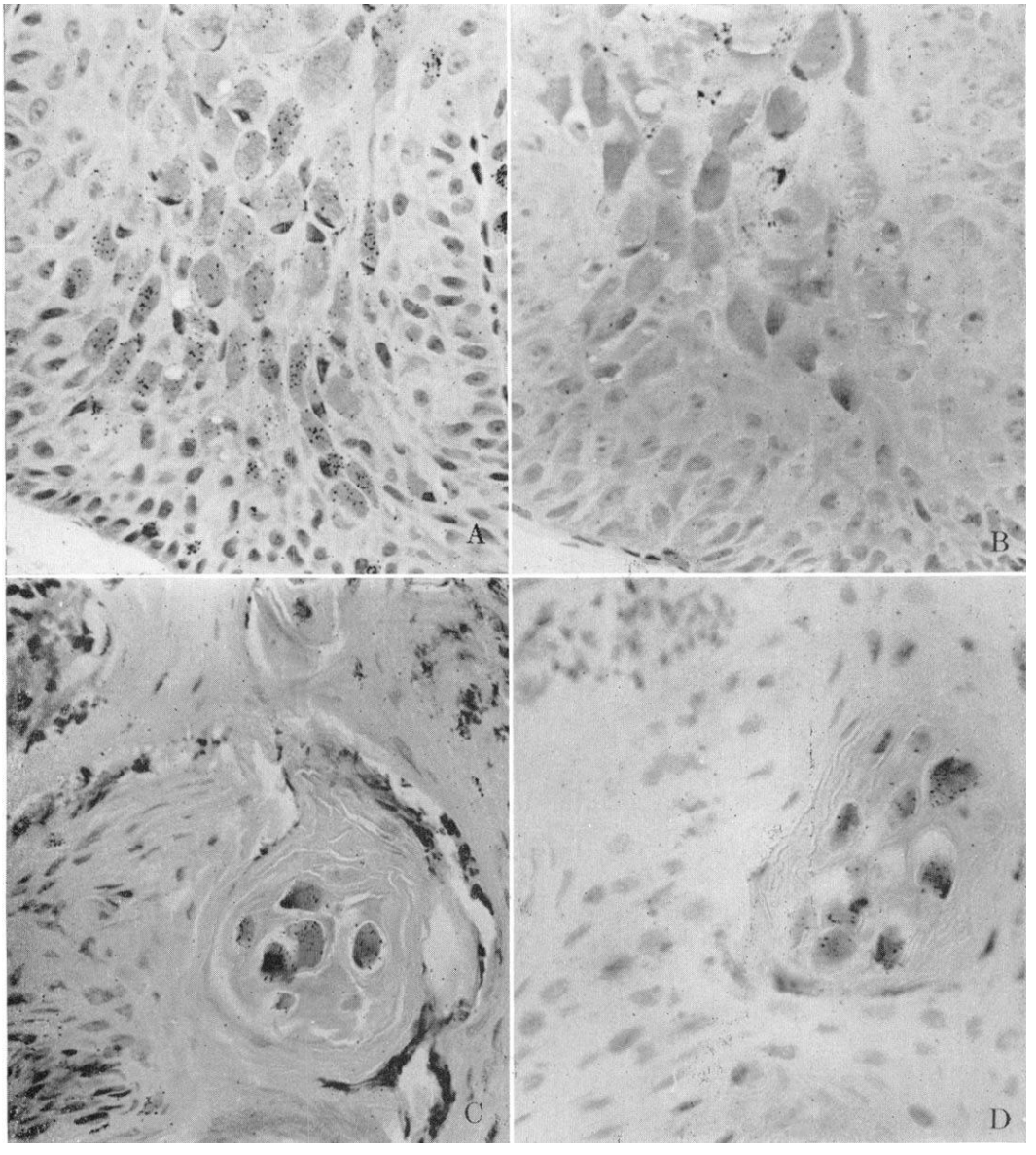


FIG. 7a. Base of molluscum lesion 40 minutes after thymidine-H^3 injection. Note typical pattern of viral and nuclear labeling.

FIG. 7b. A section similar to 7a. Partially digested with DNase and showing loss of labeled DNA from both virus and cell.

FIG. 7c. Control 9 days after thymidine-H^3 injection shows labeled inclusions far out in the horny layer. The tangential cut also reveals some nuclear labeling in crushed cells at left.

FIG. 7d. DNase treatment of a section similar to 7c removes nuclear labeling but not in the inclusion. This is similar to the situation seen during maturation of vaccinia virus. Apparently a thin layer of protein coats and protects the DNA from DNase.

parently the nucleus of infected cells retains its initial complement of DNA and is not utilized for virus formation.

At one month, although the labeled virus had long since disappeared, labeled nuclei were still observed in both infected and uninfected cells in the Malpighian layer (Fig. 10a). In addition, labeled nuclei of infected cells

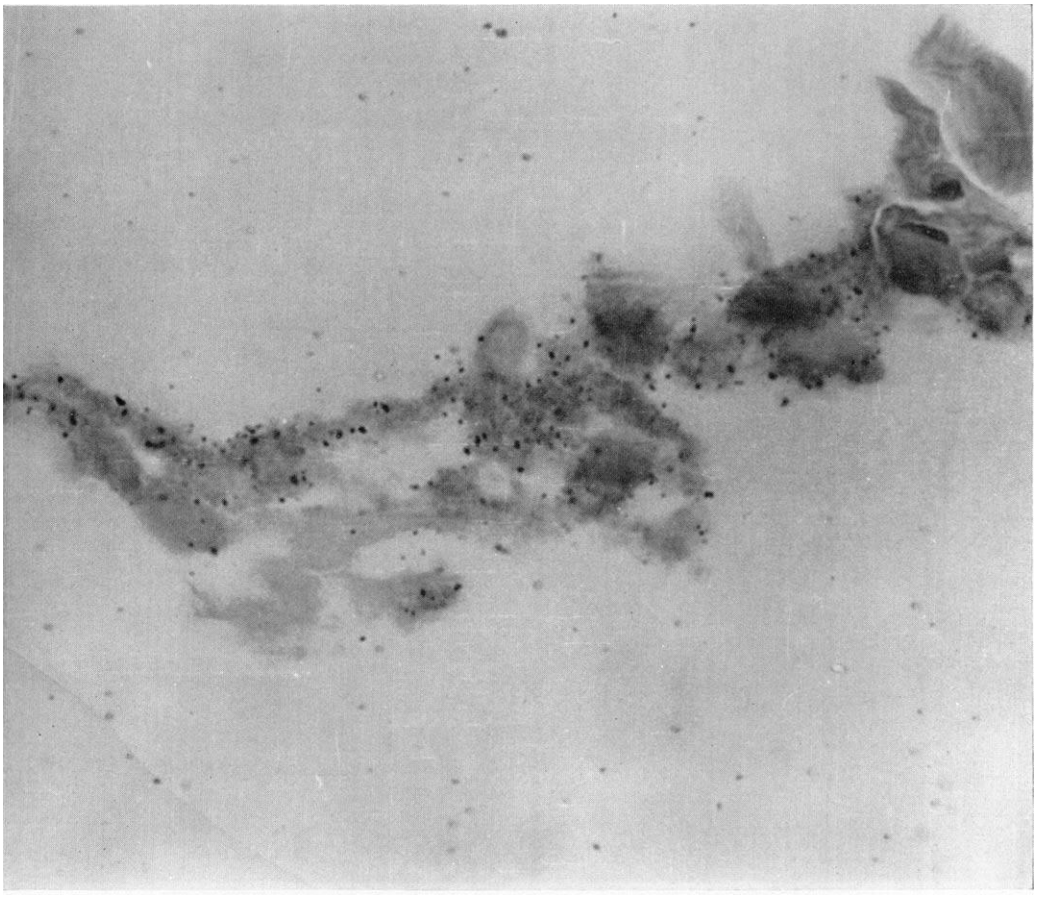


FIG. 8. Pasty material extruded from a lesion during biopsy at 9 days after thymidine-H^3 injection. The radioautograph shows labeling of cells that seem to be disintegrating. Presumably the virus is disseminated by disruption of the cells at the surface.

appeared at all levels in the horny layer (Fig. 10b). No clear-cut bands of labeled cells were noted. This reflects the random movement of cells with labeled nuclei and gives no clue as to how the cells become infected.

To summarize, the two cell populations so readily recognized in the Malpighian layer seem homogeneous in the basal layer. Although their renewal time is rapid, they move randomly in the usual way (1); nearly all take up cytidine-H^3. The cells maintain equal status for one or two layers above the base. Then suddenly cytoplasmic viral inclusions appear in some cells and their life history becomes unalterably changed. The nucleus persists but loses control; it is pushed to the edge of the cell. The virus now directs cytoplasmic RNA synthesis. It replicates almost entirely in the Malpighian layer. The other population of cells continues to make tonofibrils and keratin precursors, albeit under most unfavorable conditions. They manage, however, to form keratohyalin in an expanded granular layer and produce a faulty horny matrix with tiny birefringent bodies (Fig. 9), to support virus-laden cells during their final stages of maturation (11–14). Most remarkable, perhaps, is the discrepancy in movement of these cell types through the Malpighian layer. Once a cell becomes infected it is destined to leave the granular layer on its way to the surface within 5 days. Infected cells move as an integrated unit. Within 9 to 15 days the band of infected cells is sloughed. Uninfected cells follow a more leisurely and random route. True some reach the granular layer within 9 to 13 days (1), but most take 15 to 21 days and some remain in the Malpighian layer after a month. Virus infection then synchronizes and

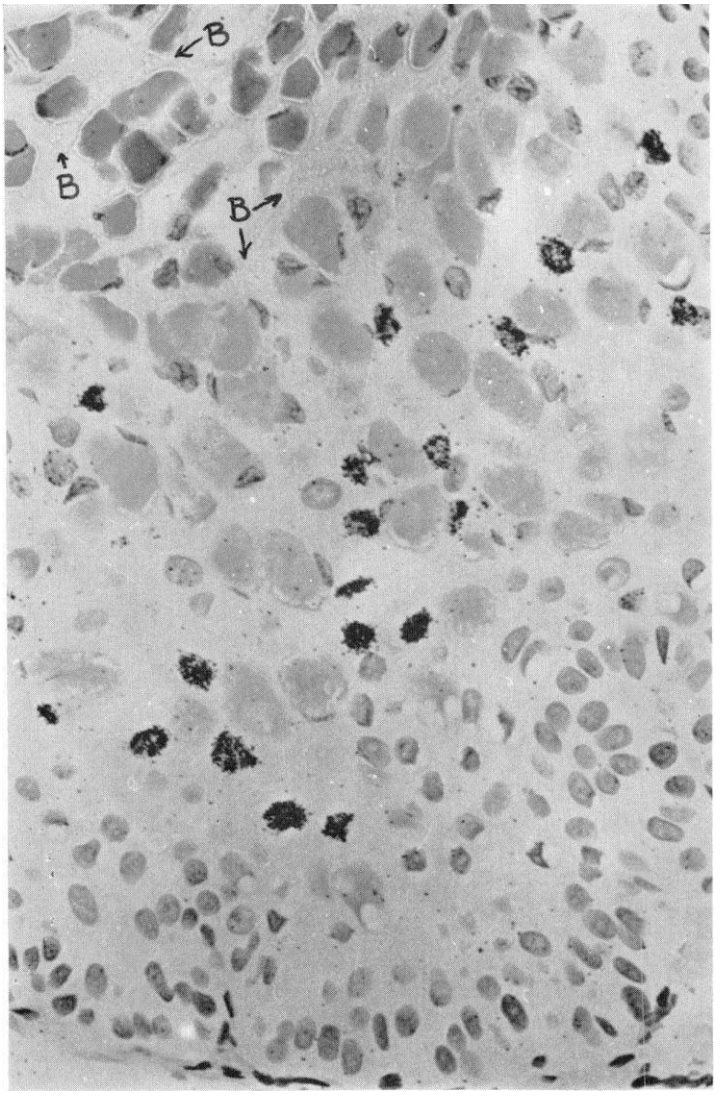


FIG. 9. Radioautograph 15 days after thymidine-H^3 injection shows heavily labeled nuclei at all levels. Note both infected and uninfected cells have labeled nuclei. Also, birefringent bodies (B) can be seen throughout the horny layer. These show very well by phase microscopy and appear similar to the bodies or vacuoles seen in the horny layer of psoriasis and other parakeratotic conditions, including warts (38–40).

speeds up the movement of infected cells without affecting the movement of uninfected cells.

DISCUSSION

These data detail some differences between infected and uninfected cells in molluscum contagiosum. A comparison of the behavior of these cell populations provides insight into the interrelations of normal epidermal cells. Cells in the epidermis are organized to withstand great mechanical stress and yet maintain a pliant configuration. Some believe the system of tonofibrils and tonofilaments with their desmosomal attachments and attachment plaques mainly account for this unique combination of strength and suppleness (17, 18). Certainly a cohesive interaction of cells is required. This has been convincingly demonstrated by microdissection of human epidermis (19). Mercer (18, 20) considers that differentiation of epidermal cells depends on cell adhesion. Kligman discusses "cohesion gradients" (21) and Wil-

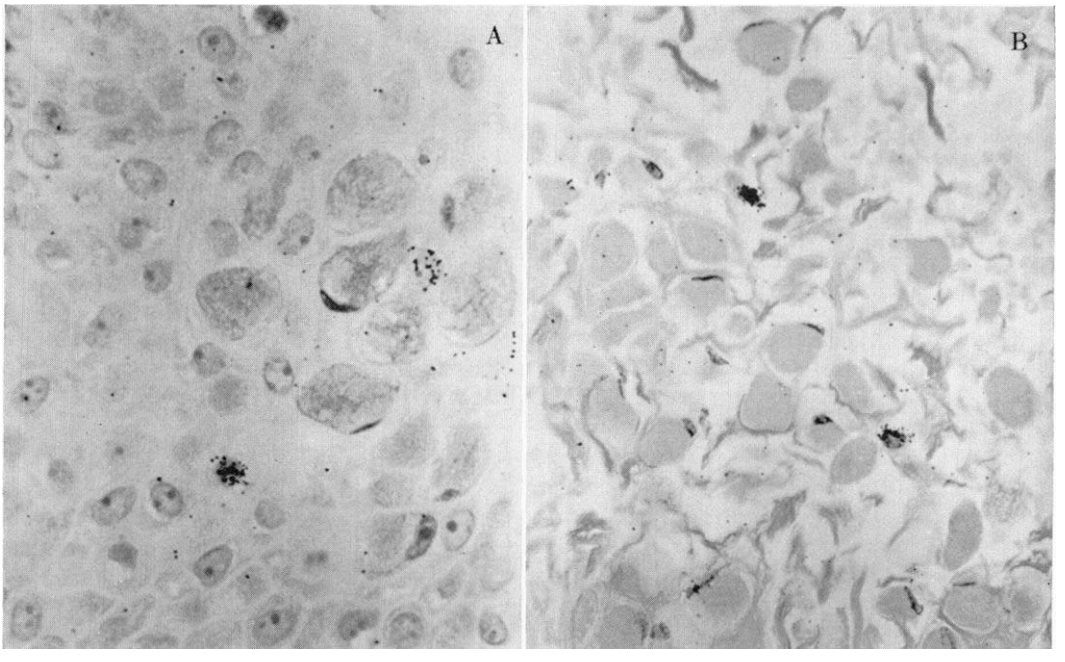


FIG. 10a. Labeled nuclei in Malpighian layer one month after thymidine-H3 injection
FIG. 10b. Labeled nuclei of infected cells far out in the horny layer one month after thymidine-H^3 injection. Note there is no loss of intensity of labeling, suggesting the virus did not use nuclear DNA for its own purposes.

gram *et al* (22) explain acantholytic blisters by loss and distortion of the desmosome-tonofilament complex. In defining epidermal cancer, Van Scott (23) emphasizes that normally keratinizing cells migrate "with, not through their neighbors" and cancer cells lose this cohesiveness. These descriptions suggest individual cells are firmly, presumably unalterably, bound to their neighbors and move to the surface as a unit. Weinstein and Van Scott recently have reaffirmed this concept (24). Aside from microdissection (19), evidence for this view rests solely on the constant finding in electron micrographs of complex attachment plaques between normal epidermal cells. But logic and a few observations belie such a rigid interpretation. Melanocytes and lymphocytes can migrate through the epidermis. Do they squeeze between attachment plaques? In discussing acantholysis and pemphigus Braun-Falco and Vogell (25) consider that epidermal cells move "singly and independent of each other" and remodel their shape as they keratinize. Thus, the desmosome-tonofilament complex of Wilgram is seen as temporary and subject to change. Christophers and Kligman (26) describe formation of horny cells in discrete columns like stacks of pancakes. To achieve this configuration they theorize that cells must remodel as they move and attachment plaques must be changing all the time. The best evidence for this concept comes from the work of Greulich (27) which we have confirmed and extended for man (1). Using thymidine-H^3 to label germinative cells he showed that cell division does not automatically lead to migration of one of the daughters. On the contrary, migration appears random. In man we found basal cells took from 9 days to 6 weeks to reach the granular layer (1). No evidence for band-like movement was observed. This implies that connections between individual cells are not permanent but must break and reform. The data from the present study offers even more dramatic support for this idea. Infected cells move as a band and leave the Malpighian layer within 5 days; uninfected cells take from 9 to more than 28 days. Charles (13) and Odland and Epstein (28) found attachment plaques between infected and uninfected cells at all layers.

At present we cannot reconcile these contradictory concepts. The meager evidence available suggests that both events occur. Epidermal

cells do not move completely free of each other. They must relate, and importantly, but connections are not permanent and must change during movement to the surface. Perhaps the "zipper-like" effect observed by Chambers and Renyi (19) and postulated by Mercer (18, 20) acts more like an escalator or cog-wheel to allow differential movement.

The virologic aspects of this paper are equally interesting. We are able to present direct evidence of virus proliferation *in vivo*, and can give some information concerning how it affects the cell. Molluscum contagiosum is generally considered a pox virus but is relegated to the unclassified group (29) because of the difficulties in growing the virus *in vitro*. Our data confirm the findings of its being a DNA virus (29). It replicates in the cytoplasm of cells in the Malpighian layer at a slow pace compared to vaccinia virus (30) over 4 to 5 days as the cell moves upward. Nuclear DNA is not utilized for synthesis of viral DNA as in some viruses (31). This speaks against the idea that pox viruses use nuclear DNA for their own purposes of synthesis (32).

Virus infection has the additional effect of shutting off transcription of RNA from the host cell genome. Vaccinia virus in culture does this within 3 hours (33) but in molluscum contagiosum it probably requires more time as some infected nuclei in the Malpighian layer incorporate cytidine-H^3. Also, it is not certain how much RNA the virus produces nor to what extent it controls viral replication (34).

The main effects of viral infection are to speed cell renewal and to synchronize the movement of infected cells. The ability to cause: 1) rapid growth, and 2) loss of contact inhibition help define cell transformation by tumor viruses (35–37). But in molluscum contagiosum the transformation never becomes malignant; loss of contact inhibition remains partial; and unregulated nuclear DNA synthesis does not occur. A type of quasi-transformation is seen. This assumes importance when we consider how the cells become infected. Are some basal cells already transformed by an inapparent infection that only manifests itself at a higher level, or do infectious particles migrate from cell to cell? The results of this study do not answer the question conclusively. No differences between basal cells were observed. They behaved like normal epidermal cells until a visible inclusion appeared. On the other hand, we could not be certain infectious particles invaded cells in the Malpighian layer. The only evidence for transfer of virus occurred in the 24 hour thymidine-H^3 injected specimens in which labeled inclusions had moved through the granular layer but no new unlabeled inclusions were seen. The overall pattern of movement suggests that labeled virus was transferred to newly formed inclusions during this period. However, it seems curious we did not then see a trail of diminished labeling as the cells moved outward. Further work is required to settle the issue.

SUMMARY

Virus-infected and normal epidermal cell kinetics were determined in molluscum contagiosum by intradermal injections of thymidine-H^3 and cytidine-H^3 to label nuclear and viral DNA and RNA.

In the basal layer the germinative cell renewal time proved more rapid than normal (average 3.4 days). Viral inclusions became labeled only in the Malpighian layer. These labeled infected cells moved upward in a band leaving the granular layer within 5 days and the horny layer in 9 to 15 days. In contrast, labeled nuclei of uninfected cells required 9 to 30 days to reach the granular layer.

Virus replication occurs in the Malpighian layer. Nuclear DNA is not utilized for virus production. All inclusions in the Malpighian layer contain labels for 24 but not 48 hours, suggesting that infectious particles are transferred from cell to cell. Infection shuts off transcription of RNA from the host cell genome. Some RNA is formed in the cytoplasm, presumably under viral direction.

The results indicate two populations of cells exist: 1) infected cells which move rapidly and as a unit; and 2) uninfected cells which move more slowly and apparently randomly. The findings are discussed in terms of how normal epidermal cells relate to each other during differentiation.

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